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Abstract

Effects of Gabexate mesilate (GM) (([ethyl-4-(6-guanidino hexanoyloxy) benzoate] methane sulfonate)), a protease inhibitor, on the activities of catalase in liver, erythrocytes and reticulocytes from acatalasemic mice were examined. Preincubation without GM at 37 degrees C for 160 min lowered the catalase activities of liver, erythrocytes and reticulocytes from acatalasemic mice, to 24%, 40% and 10% of the initial levels, respectively. But, preincubation with GM at 37 degrees C for 160 min delayed the rapid decrease in activities of residual catalases in the liver, erythrocytes and reticulocytes of acatalasemic mice to 65%, 93% and 85% of the initial values, respectively. At 20 degrees C or below, no reduction in catalase activity of reticulocytes from acatalasemic mice occurred with or even without GM. At pH 5.0, the decrease in catalase activity of acatalasemic mice was small both in the presence and the absence of GM. In the alkaline range, the reduction in the enzyme activity of the mutant mice without GM was enhanced with increase in pH values up to 8.5. But the presence of GM during preincubation at pH 7.5, retained the catalase activity of acatalasemic mice, to 64% of the activity at pH 6.5. These data suggest that some factors affected by GM, might be responsible for the low stability and activity of catalase in the acatalasemic mice.

KEYWORDS: acatalasemic mouse, residual catalase, Gabexate mesilate, protease inhibitor

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Effect of a Protease Inhibitor on the Stability of Catalase in Liver and Blood from Acatalasemic and Normal Mice

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Effects of Gabexate mesilate (GM) (([ethyl-4-(6-guanidino hexanoyloxy) benzoate] methane sulfonate)), a protease inhibitor, on the activities of catalase in liver, erythrocytes and reticulocytes from acatalasemic mice were examined. Preincubation without GM at 37°C for 160 min lowered the catalase activities of liver, erythrocytes and reticulocytes from acatalasemic mice, to 24%, 40% and 10% of the initial levels, respectively. But, preincubation with GM at 37°C for 160 min delayed the rapid decrease in activities of residual catalases in the liver, erythrocytes and reticulocytes of acatalasemic mice to 65%, 93% and 85% of the initial values, respectively. At 20°C or below, no reduction in catalase activity of reticulocytes from acatalasemic mice occurred with or even without GM. At pH 5.0, the decrease in catalase activity of acatalasemic mice was small both in the presence and the absence of GM. In the alkaline range, the reduction in the enzyme activity of the mutant mice without GM was enhanced with increase in pH values up to 8.5. But the presence of GM during preincubation at pH 7.5, retained the catalase activity of acatalasemic mice, to 64% of the activity at pH 6.5. These data suggest that some factors affected by GM, might be responsible for the low stability and activity of catalase in the acatalasemic mice.

Key words : **acatalasemic mouse, residual catalase, Gabexate mesilate, protease inhibitor**

Catalase (EC 1. 11. 1. 6; hydrogen-peroxide: hydrogen peroxide oxidoreductase) catalyses the breakdown of H_2O_2 to O_2 and H_2O. This enzyme plays a part in protecting cells from the toxic effects of H_2O_2 generated during cellular metabolism, oxygen radical and H_2O_2 generated by superoxide dismutase. Takahara first reported a human case of acatalasemia which exhibited a deficiency of blood catalase activity and oral gangrene (1). Ogata and Takahara demonstrated residual catalase activity in acatalasemic human blood (2). Ogata *et al.* showed that the residual catalase of Japanese-type acatalasemic patients was indistinguishable from the enzyme of normal subjects in properties such as isoelectric point (3), subunit size (4), heat stability and immunological characteristics (5). Recently Wen *et al.* (6) showed that immunoreactive catalase protein was severely reduced in Japanese-type acatalasemia. This reduction of catalase protein was due to the serious decrease in catalase mRNA. Then they

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determined the nucleotide sequence of the mutant gene for all exons, exon/intron junctions, and 5' and 3' flanking regions, obtaining seven base differences between the two genes. They demonstrated that a guanine to adenine replacement at the fifth position in the 4th introne was the causal mutation of Japanese-type acatalasemia. This substitution severely limited the correct splicing of the RNA product (7).

An acatalasemic mouse strain, Cs^b, was first described by Feinstein *et al.* (8). The residual catalase in the erythrocytes of acatalasemic mice has more heat labile (9, 10) and exhibits higher isoelectric point on agarose gel electrophoresis than the normal enzyme (3). Shaffer *et al.* (9), have reported that acatalasemic mice have a normal level of catalase mRNA indistinguishable in size from normal. These observations suggested that a mutation in the catalase gene caused defective mRNA translation and/or formation of more unstable catalase protein which more rapidly turned over in acatalasemic mice. But the molecular and genetic basis of the acatalasemic catalase mutation in mice has not yet been fully elucidated. In this study we have tested whether the intracellular proteases are responsible for the rapid degradation of abnormal catalase in acatalasemic mice. But the responsible enzymes or factors are still unknown at present. Therefore, we tried to add Gabexate mesilate (GM) (([ethyl-4-(6-guanidino hexanoyloxy) benzoate] methane sulfonate)) (11), which had inhibitory effect on various serine proteases, to the preincubation mixture. We have demonstrated that the presence of GM slowed down the rapid reduction in activities of residual catalase from acatalasemic liver, erythrocyte and reticulocyte during preincubation under various conditions with respect to time, temprature and pH.

Materials and Methods

Animals and Reagents

Male acatalasemic (C3H/AnL Cs^bCs^b) and wild type (C3H/AnL Cs^aCs^a) mice about 30 g were used throughout the experiments. All chemicals used were reagent grade. Gabexate mesilate (GM) ([ethyl-4- (6-guanidino hexanoyloxy) benzoate] methane sulfonate)) was kindly given by Ono Pharmaceutical Co.

Preparation of Reticulocytes

Reticulocytosis was induced in mice by daily subcutaneous injection of 1 % phenylhydrazine hydrochloride (pH 7.2). About 0.01 ml of this solution per g body weight was injected for 3 days. Blood was drawn from the abdominal aorta of mice, into heparinized tubes under light anaesthesia on the 5th day from the first injection. The blood preparations showed reticulocyte count of about 60 %.

Effects of GM on the Decrease of Catalase Activity in Liver Homogenate and Blood Lysate

Measurement of catalase activity. For the assay of liver catalase, liver was homogenized with 12.5 mM potassium phosphate buffer (pH 6.8) and diluted 500 or 1000 times in acatalasemic or normal mice, respectively. The blood was diluted with cold distilled water and phosphate buffer (final concentration 12.5 mM) to 10 or to 125 fold in acatalasemic or in normal mice, respectively. Then, blood lysate was centrifuged at 3,000 rpm for 30 min at 0°C. After centrifugation catalase activity was determined by the perborate method. This method involved a 5 min incubation at 20°C (12). The concentration of protein was determined by the biuret reaction (13).

Time dependent decrease. Liver homogenate, erythrocyte lysate and reticulocyte lysate were preincubated at 0 or 37°C, pH 6.8 for 40, 80, 160 min. At each time 0.1 ml of the samples were pipetted directly into tubes of perborate (pH 6.8) for catalase assay. GM was added at the concentration of 1 mg/ml to the preincubation and the assay mixtures.

Temperature dependent decrease. Reticulocyte lysate was preincubated for 80 min at 0, 20, 30 and 37°C, pH 6.8 with or without GM. Then the remaining catalase activities were assayed.

Hydrogen-ion concentration dependent decrease. Reticulocyte lysate was preincubated for 10 min at 37°C, at various pH, with or without GM. Then catalase activities was assayed. Acetate buffer was used at pH 5.0. At pH 6.5 and 6.8, phosphate buffer was used. At pH 7.5 and 8.5, Tris · HCl buffer was used. All buffers were prepared at concentrations of 12.5 mM.

Effect of GM on catalase activity. GM was added to the reticulocyte lysate at final concentrations of 1 mg/ml or of 2 mg/ml. Reticulocyte lysate was preincubated in 12.5 mM Tris · HCl, pH 7.5, at 37°C for 10 min. Then

catalase activities was assayed.

Results

Effects of GM on the time dependent decrease of catalase activity. Figs. 1, 2 and 3 show the changes of time dependent decrease in catalase activity of liver homogenate (Fig. 1), erythrocyte lysate (Fig. 2) and reticulocyte lysate (Fig. 3) from acatalasemic and normal mice at 37° C, pH 6.8. The initial activities of catalase of reticulocyte lysate from acatalasemic mice remained unchanged when they were preincubated at 0°C in the presence or absence of GM for periods up to 160 min. The catalase activity of reticulocyte lysate from normal mice by the addition of GM, preincubated at 37°C for 160 min was 1.5 times higher than that of the initial value (Fig. 3). Whereas, the catalase activities of liver, erythrocyte and reticulocyte of acatalasemic mice without GM were markedly reduced within 160 min to 24 %, 40 % and 10 % of the initial values, respectively. With the addition of GM, at a concentration of 1 mg/ml, the rate of decrease in apparent activities of catalase in liver homogenate, erythrocyte lysate and reticulocyte lysate of acatalasemic mice, slowed down to 65 %, 93 % and 85 % of the initial values, respectively. The inhibitory effect of reticulocyte on catalase activity was greater than that of erythrocyte in acatalasemic mice.

Effect of GM on temperature dependent decrease of catalase activity. Fig. 4 shows the effect of the temperature during preincubation on catalase activities of the mutant and normal mice.

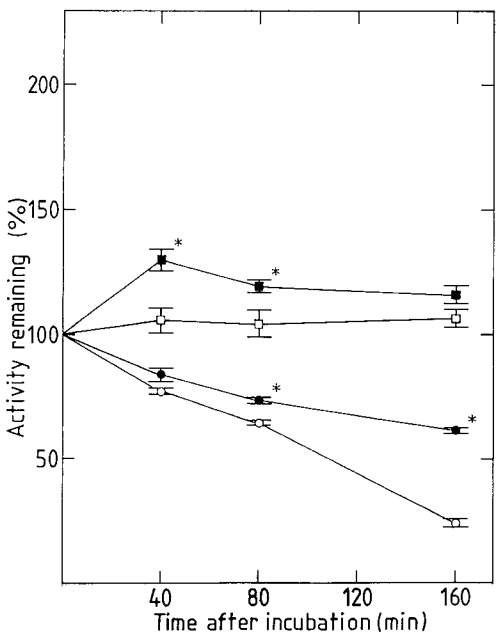

Fig. 1 Changes with time in the activities of catalase in livers from acatalasemic and normal mice. -○-: acatalasemic; -●-: acatalasemic (+ GM); -□-: normal; -■-: normal (+ GM). Vertical bars show Means ± S.E. of four animals. *; Significantly different ($p < 0.05$) from each control (without GM) value. For abbreviation of GM see text.

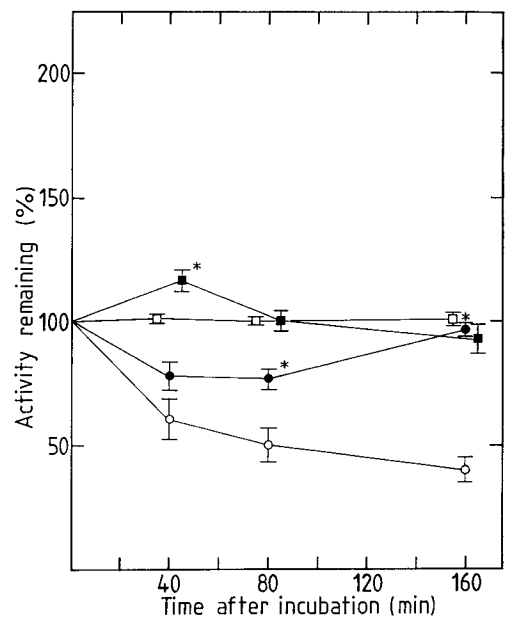

Fig. 2 Changes with time in the acttivities of catalase in erythrocyte lysate from acatalasemic and normal mice. -○-: acatalasemic; -●-: acatalasemic (+ GM); -□-: normal; -■-: normal (+ GM). Vertical bars show Means ± S.E. of four animals. *; Significantly different ($p < 0.05$) from each control (without GM) value. For abbreviation of GM see text.

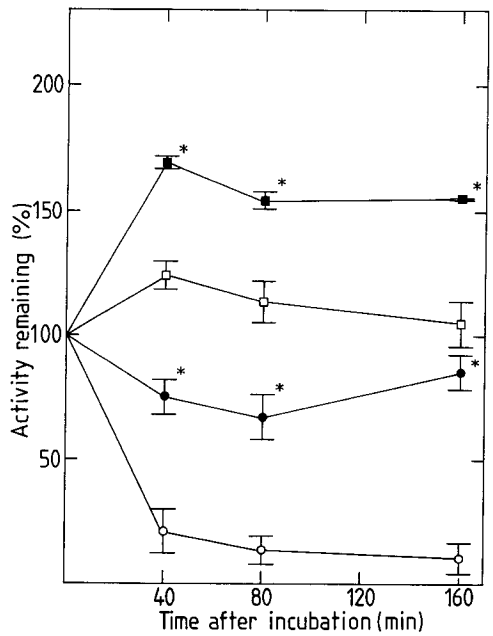

Fig. 3 Changes with time in the activities of catalase in reticulocyte lysate from acatalasemic and normal mice. -○-: acatalasemic; -●-: acatalasemic (+ GM); -□-: normal; -■-: normal (+ GM). Vertical bars show Means ± S.E. of four animals. *; Significantly different ($p < 0.05$) from each control (without GM) value. For abbreviation of GM see text.

At 20°C or below, no inactivation of catalase activity of reticulocytes of mutant mice occurred. At 37°C, the inactivation of catalase in acatalasemic mice strongly proceeded without GM. But, the catalase activity of reticulocytes at 37°C was almost restored by the addition of GM.

Effect of GM on pH dependent decrease of catalase activity. Fig. 5 demonstrates the pH dependence of catalase activity with or without GM, in which the relative activity at pH6.5 was taken as unity. At pH5.0, the decrease in catalase activity in acatalasemic mice is small, but in the alkaline range, above pH7.5 it became evident. Percent inhibition without GM, at pH 7.0 in the mutant mice is extrapolated to be about 70%. The optimum pH of reticulocyte catalase in acatalasemic mice was around pH6.5, and in normal mice it is pH7.5. Therefore, catalase of reticulocyte in acatalasemic mice was more resistant to acidic denaturation than that of normal. Inactivation of catalase activity in alkaline range without GM might be caused by the dissociation of catalase into subunits (18, 19). But the decrease in activity of acatalasemic catalase at pH 7.5 was prevented in part by the addition of GM.

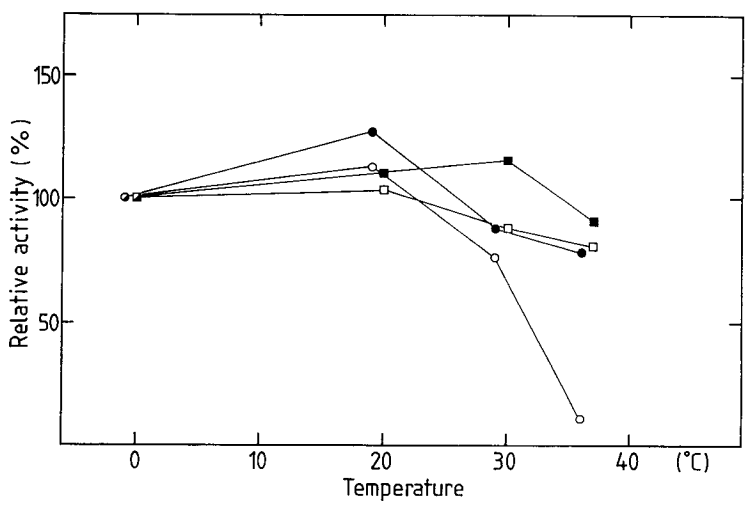

Fig. 4 Temperature dependence of inactivation of catalase in reticulocyte lysate from acatalasemic and normal mice. -○-: acatalasemic; -●-: acatalasemic (+ GM); -□-: normal; -■-: normal (+ GM). Each point represents mean of two animals. For abbreviation of GM see text.

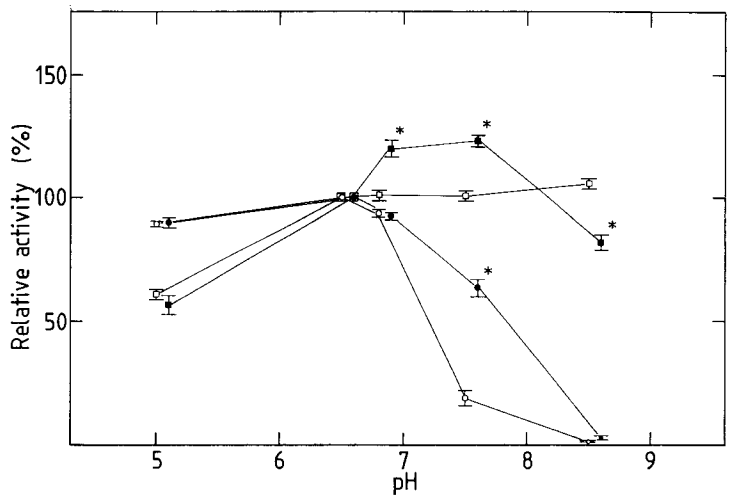

Fig. 5 pH Dependence of inactivation of catalase in reticulocyte lysate of acatalasemic and normal mice. -○-: acatalasemic; -●-: acatalasemic (+ GM); -□-: normal; -■-: normal (+ GM). Vertical bars show Means ± S.E. of four animals. *; Significantly different ($p < 0.05$) from each control (without GM) value. For abbreviation of GM see text.

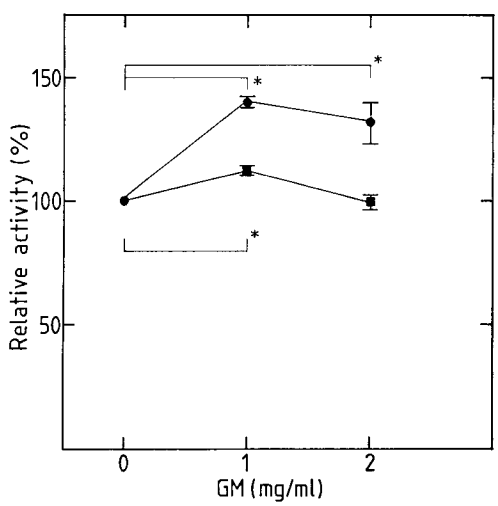

Fig. 6 Dose-dependent inactivation of catalase in reticulocyte lysate of acatalasemic and normal mice. -●-: acatalasemic (+ GM); -■-: normal (+ GM). Vertical bars show Means ± S.E. of four animals in normal and of three animals in acatalasemia. * Significantly different from ($p < 0.05$) each initial (without GM) value. For abbreviation of GM see text.

Hence, the inactivation of acatalasemic catalase at pH7.5 without GM might be caused by both proteolysis and dissociation into subunits.

Effect of GM on catalase activity. Fig. 6 shows dose-dependent effects of GM on catalase activities. The inactivation of catalase in reticulocyte lysate of acatalasemic mice was protected at the concentration 1 mg/ml of GM, then it reached a plateau.

Discussion

Iden (14) recently showed that the decrease in activity of reticulocyte catalase during maturation to erythrocyte was higher in acatalasemic mice than that in normal mice. The reasons for low stability and low level of catalase in acatalasemic mice are still unknown.

Shaffer *et al.* (9) recently studied the genetic control of gene expression by analysing catalase transcription and translation products from the

tissues of acatalasemic and control mice. They showed reduced levels of immunologically reactive catalase protein of acatalasemic kindney and hemolysate by immunoblot analyses. But their findings account for the tissue-specific reduction of catalase activity in acatalasemic mice.

When rat liver catalase was purified in the absence of protease inhibitor, the subunit was little smaller in size than that purified in the presence of inhibitors (15, 16). In the first experiment we have examined whether the decreased stability of catalase in acatalasemic mice is caused by the difference in susceptibility to proteases. We have shown that GM delayed the inactivation of catalases in liver homogenate of acatalasemic mice during preincubation at 37°C, pH 6.8. In the blood samples, GM also slowed down the inactivation of catalase in erythrocyte and reticulocyte lysate. The extent of inhibition of catalase activity in reticulocyte was greater than that in liver or erythrocyte. Therefore, the extent of the reduction in the catalase activity in acatalasemic mice appeared to be derived from the differences in the properties and the amounts of inactivator and/or coexisting proteins. Inactivation of catalase activity in reticulocytes was strongly dependent on pH and temperature as shown in Figs. 4 and 5. Muller *et al.* (17) have reported that the rate of proteolysis of rabbit reticulocytes was only 33–10 % of that at 37°C, at temperatures of 27°C or below. The optimum pH of protein breakdown existed between pH 7.6 and 7.9, in rabbit reticulocytes. Samejima *et al.* (18) have reported that bovine liver catalase dissociated partially between pH 3 and 4 and completely below pH 3.0 into 1/2-size subunits by acid denaturation. Denaturation with alkali solution of pH values exceeding 9.9, for 30 min revealed that the native catalase molecule was split into subunits (19, 20). Fig. 5 showed that the catalase of reticulocyte of acatalasemic mice was less resistant to alkaline denaturation than that of normal. Denaturation with alkali during short-term preincubation within 10 min had no effect on the activity of normal catalase without GM but in the presence of GM, showed a inhibitory effect only when at pH 8.5. Alternatively, in acatalasemic mice, the inactivation of catalase activity took place rapidly with alkali during preincubation without GM. But, at pH 7.5, this inactivation was partly restored by GM (Fig. 5). Furthermore, Fujimura (21) reported that the alkaline stability of acatalasemic catalase in the anemic hemolysate at pH 8.0 was lower than that of its crude catalase solution. Therefore, the inactivation of catalase activity of reticulocyte in acatalasemic mice at pH 7.5 might be caused by the proteolysis rather than by the dissociation of catalase into subunits.

Genetic defect producing low stability of catalase in acatalasemic mice might be caused by the formation of unstable catalase. These abnormal proteins might be more susceptible to proteases, thereby bringing about the decrease in activity of reticulocyte catalase in the process of maturation (22). The determination of the sequence of the catalase gene of acatalasemic mice might be crucial to clarify the abnormal sensitivity of acatalasemic catalase to denaturation. Other than catalase gene mutation, our data suggested that inactivation (including proteases) or stabilizing factors affected by GM, might be responsible for the low stability and activity of catalase in the acatalasemic mice.

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